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The Enzymatic Degradation of Chondromucoprotein by Cell Free Extracts of Human Cartilage

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Until recently, the initial changes in osteoarthritis have been considered to be essentially a mechanical degenerative process and beyond efforts at prevention or control. The sequence of gross and microscopic changes in articular cartilage which occurs in this condition is well known. The hyaline cartilage, normally a pearly grey, smooth and glistening substance, becomes opaque, dull and pitted with yellow discoloration. The small grooves and pits which have formed in these areas enlarge producing deep clefts and the tissue loses its resilience, upon which the nutrition of chondrocytes is dependent. There is a general paucity of cellular elements with occasional clusters of proliferating chondrocytes. Histologically the articular surface shows fibrillation with loss of mucopolysaccharide as evidenced by an absence of metachromatic staining of these areas with toluidine blue. This process leads inevitably to erosion of the cartilage exposing the pain sensitive subchondral bone. At the margins of the articular surface spur formation takes place. These are initially cartilaginous, but become osseous by enchondral ossification. Cartilaginous loose bodies or "joint mice" are often found in the joint space. (1)

The biochemical alterations which parallel these changes are increasingly the subject of numerous investigations. In 1944, Hirsch, investigating the pathogenesis of chondromalacia of the patella, found that the chondroitin sulfate (CSA) content of the involved cartilage was often decreased in samples which grossly showed no fissures. (2) Kellgren, in a review of the problem, proposed that a decrease in the polysaccharide content of carti-

lage resulted in a breaking up and disintegration of unsupported collagen fibrils by wear and tear.[3] B. F. Matthews, investigating the collagen/CSA ratio in fibrillar cartilage from osteoarthritic joints, found that the ratio was greater in fibrillated than in normal cartilage reflecting a loss of CSA of greater magnitude than loss of collagen.[4]

The enzymatic degradation of chondromucoprotein (CMP) with splitting off of CSA may be involved in the biochemical changes noted. Trypsin and testicular hyaluronidase have been shown to decrease the viscosity of CMP solutions, but lysozyme has no effect.[5] Intravenous papain causes the degradation of CMP in vivo, resulting in loss of basophilic and metachromatic staining of hyaline cartilage.[6] Tsaltas has reported a depletion of CMP and CSA from rabbit cartilage after the administration of intravenous papain. The loss of CSA was found to be greater than the CMP loss.[7] Incubation of cartilage strips with bovine plasmin results in a liberation of considerable CSA into the incubation medium as compared to control samples and incubation media to which antiplasminogen had been added.[8] Bacterial enzymes which degrade CMP have also been reported.[9] Recently Ziff et al. have reported the enzymatic degradation of the CSA-protein complex by leukocytic and synovial extracts. Activity of the synovial extracts was clearly related to tissue content of polymorphonuclear leukocytes in these rheumatoid joints. (10) The decrease in ester sulfate content of articular cartilage strips incubated in Tyrode's solution as a control was found to be greater than that produced by human plasmin during incubations of 15 hours

duration reported by Chrisman et al. [11]

The latter findings raised the question of the presence of an enzyme in human articular cartilage itself which could cause the degradation of the protein-CSA complex. It is the purpose of this paper to investigate the possible presence of such an enzyme in human articular cartilage by incubating CMP with cell free extracts of normal and pathological cartilage and measuring the reaction by viscometry.

Methods

Chondromucoprotein (CMP) was prepared from calf nasal cartilage according to the method of Shatton and Schubert. [5] Solutions of varying concentration were prepared with saline - phosphate buffer, pH 7.0.

Cell free extracts of surgical specimens were prepared in the following manner. The cartilage samples were weighed and thoroughly homogenized in 0.9N NaCl (1 gram to 45 ml.) in a Virtis-45 machine at 4°C. These homogenates were centrifuged at 4000 RPM for 45 minutes. The cell free supernatants were retained for the incubations.

The degradation of chondromucoprotein by these extracts was studied by viscometry. Six ml. of 1% chondromucoprotein and two ml. of each extract were incubated in Cannon-Fiske #100 viscometers at 37°C for three hours. Efflux time determinations were made at 0,5,10,15 and 30 minutes and at succeeding 30 minute intervals to three hours. Per cent initial relative viscosity was calculated by the formula; %initial relative viscosity = $\frac{to-tx}{tx}$ where t_o is the efflux time at the initial read-

ing and t_x is the efflux time at any succeeding determination.

The enzymatic nature of the reaction was investigated in several ways. Trace amounts of various enzyme inhibitors including $HgCl_2$, NaCN and IAA were added to the reaction mixture. The extracts were heated to 60°C for 10 minutes before incubation with chondromucoprotein. Finally the concentration of CMP was varied from 0.75 to 1.5% and its effect on reaction rate recorded.

A study of the reaction products was also carried out. After the reaction was terminated by freezing, the reaction mixture was filtered through an ultra fine fritted glass bacterial filter ("Pyrex" Brand Chemical Glass, #33990, UF porosity) to remove protein.[12] The filtrate was spotted (0.04ml.) on #1 Whatman paper and electrophoresis carried out with chondroitin sulfate A and C, prepared from calf nasal septum and steer tendon respectively by the method of Einbinder and Schubert, as control spots.[13] The filtered reaction mixture was also analyzed for the presence of amino acids and sugars by paper chromatography with a filtrate of 1% CMP solution incubated alone for three hours with buffer serving as a control. Chromatographic technique for sugars involved spotting of 50 microliters on Whatman #4 paper and a solvent prepared as follows: 600ml. n-butanol, 165ml. ethanol, 400ml. distilled water and 10ml. NH_3 (3.2ml. conc. NH_3 (w/v) made up to 100 ml. with water). Chromatography was run for 40 hours and sugars were stained by a process involving the use of aniline - phthalate spray and heating to 100 - 105 C for 10 minutes. Identification of amino acids involved a two phase process in which 50 micro-

liters were spotted on Whatman #4 paper. Water saturated phenol with 6 - 8 drops of concentrated ammonia and a trace of NaCN was used as solvent in the first phase. Second phase solvent was prepared from 600 ml. collidine, 600 ml. 2,4 - lutidine and 1000 ml. distilled water with 6 - 8 drops of diethylamine added. Paper was stained with 0.15% ninhydrin in acetone.

Results

I Effect of Extracts on Chondromucoprotein Viscosity

CMP in 1% solution was incubated with neutral saline-phosphate buffer for three hours as a control. There was no decrease in the viscosity of the solution.

Six normal cartilage extracts showed an average viscosity reducing activity of 6.4% with a range of 1.9 to 10.9% (table 1).

Table 1

Viscosity Reducing Activity of Normal Cartilage Extracts

sample #	age	% decrease in relative viscosity (3 hours)
1	17	9.3
3	18	2.4
7	9	7.7
8	7	1.9
17	17	10.9
22	30	6.2

Eight pathological samples were obtained from osteoarthritic joints. Of these, five were from fibrillated areas and

three were spur cartilage. The five chondromalacic samples showed an average viscosity reducing activity of 9.9% with a range of 2.6% to 19.1%. The spur cartilage on the other hand showed an average viscosity reducing activity of 3.1% with a range of 2.7% to 3.6%. (table 2) Although the chondromalacic samples showed an activity which was significantly greater than that of spur cartilage, they did not show much greater activity than the normal samples.

Table 2

Viscosity Reducing Activity of Hypertrophic Arthritic Cartilage Extracts

sample #	source	age	% decrease in relative viscosity (3 hours)
2	C *	35	19.1
6	C	20	2.6
14	C	30	13.0
15	C	50	9.6
20	C	19	5.4
4	S *	62	2.7
5	S	27	3.3
18	S	46	3.6

* C = Chondromalacia; * S = Spur cartilage

Several other tissues were studied including loose bodies and one chondrosarcoma. Extracts of three loose bodies showed an average viscosity reducing activity of 3.1. The chondrosarcoma extract had a 17.1% viscosity reducing activity in three hours (table 3).

Table 3

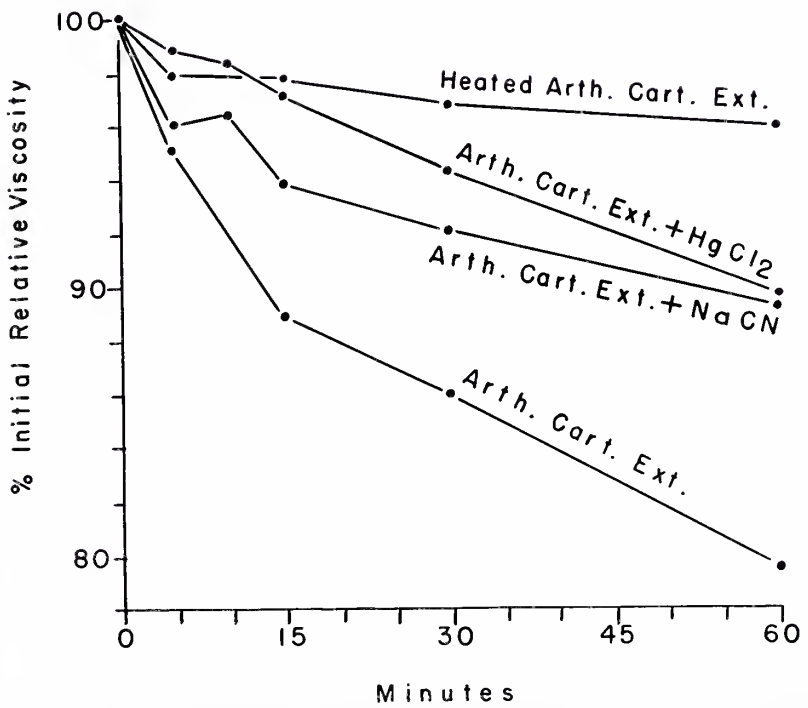
Viscosity Reducing Activity of Other Pathological Extracts

Sample	Source	% decrease in relative viscosity (3 hours)
9	Loose Bodies	3.8
24	Loose Bodies	2.8
36	Loose Bodies	2.7
11	Chondrosarcoma	17.1

II Enzyme Characteristics.

The most active osteoarthritic extract was used in a series of experiments to demonstrate the enzymatic nature of the reaction. Enzyme inhibitors in trace amounts were added to the reaction mixture. NaCN and $HgCl_2$ reduced the activity of the extract by about 50%. IAA, however, had no effect. Heating of the extract prior to incubation with CMP destroyed its activity almost completely (figure 1).

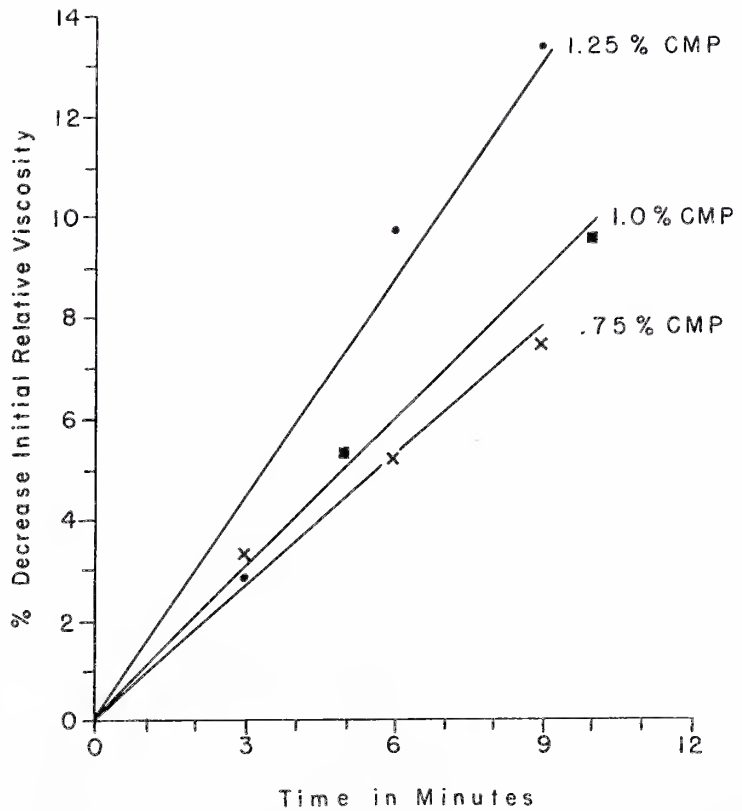
Figure 1



Effect of Heat, $HgCl_2$ and NaCN on viscosity reducing activity of cell free human cartilage extracts.

The effect of changes in enzyme concentration, pH and temperature have not been studied. However, the effect of change in substrate concentration was studied by varying the concentration of chondromucoprotein from 0.75 to 1.25% while holding enzyme concentration constant. As is illustrated in figure 2 an increase in chondromucoprotein concentration resulted in a corresponding increase in reaction rate, a pattern typical of any chemical reaction.

Figure 2



Effect of changes in CMP concentration on rate of viscosity reduction by cell free human cartilage extracts.

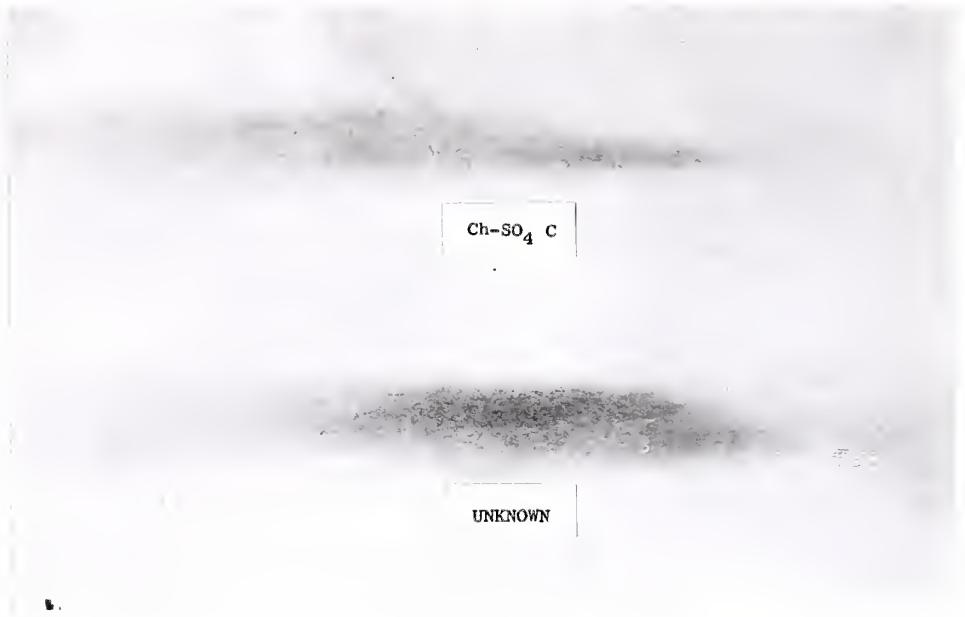
The reaction rate using 1.5% chondromucoprotein was identical to that of the 1.25% reaction suggesting that the reaction had reached a maximum velocity which could not be changed by further increase in substrate concentration. This phenomenon is characteristic of those chemical reactions which are enzymatic in nature and is due to the limited rate of formation of the enzyme - substrate complex. Further study of this problem was curtailed by our inability to prepare a suitable solution of chondromucoprotein with a concentration greater than 1.5%. We feel that the evidence is sufficient to indicate that the decrease in relative viscosity of chondromucoprotein solutions which we have observed is due to enzyme activity.

III Analysis of Reaction Products

The problem of site of action of the enzyme or emzymes involved in the process of chondromucoprotein degradation was approached by an analysis of reaction products. There were three main possibilities: 1) the enzyme was proteolytic in nature, degrading the protein to amino acids and peptides, 2) it was a chondroitinase degrading the CSA portion of the complex; or, 3) it was an enzyme splitting off chondroitin sulfate intact from the protein.

After ultrafiltration of the reaction mixture to remove residual protein, the filtrate was electrophoresed with chondroitin sulfate A and C as control spots and also subjected to paper chomatographic analysis. Electrophoresis of the filtrate revealed a large metachromatic spot with a mobility identical to that of chondroitin sulfate C, but slightly less than that of chondroitin sulfate A, figure 3 and 4.

Figure 3



Electrophoresis of protein free filtrate of reaction mixture and chondroitin sulfate C. Acetate buffer, pH 4.3.

Figure 4

Electrophoresis of protein free filtrate of reaction mixture and chondroitin sulfate A. Acetate buffer, pH 4,3.

As the size and metachromaticity of the spot were notable, it was felt that undegraded CSA was a major reaction product. Chromatography was carried out to investigate the possibility that there might be some mucopolysaccharide degradation or proteolytic activity present in the cartilage extracts. Neither amino acids, peptides or sugars were demonstrable in the filtrate by this method. It is felt that CSA is being split from the protein at or very near the protein-chondroitin sulfate bond releasing CSA intact or perhaps with a small polypeptide attached.

Discussion

Although the intermediate steps in the synthesis of the CSA molecule through the UDPG system and "active" sulfate have been well demonstrated, (14, 15, 16,) the sequence of steps by which the ground substance of mammalian cartilage is degraded is poorly understood. In approaching this problem it is necessary to determine the initial biochemical changes which occur in normal and pathological states. There is neither histological nor biochemical evidence that degradation of collagen is involved in the early steps of this process. There has been no demonstration of a collagenase which is functional at neutral pH in mammalian tissues. The work of Matthews has demonstrated a loss of CSA rather than collagen in fibrillated cartilage from osteoarthritic joints. The problem then clearly involves the catabolism of the other major component of the ground substance, chondromucoprotein.

There are a variety of known enzymes which are capable of degrading CMP, but which can not logically be indicted as the agent involved in the degeneration of the ground substance in osteoarthri-

tis. The various plant and bacterial enzymes, of which papain is the most noteworthy, simply are not present in the sterile joints. There are insufficient polymorphonuclear leukocytes involved in the osteoarthritic process to incriminate the leukocytic enzyme which Ziff has proposed as a factor in the destruction of articular cartilage in rheumatoid arthritis. The proposal has been made that plasmin, a proteolytic agent involved in fibrinolysis, may be involved in CMP catabolism in the arthritic joint. Certainly this enzyme could gain access to the joint surface as a result of trauma and hemorrhage into the synovial space. It has been recently shown that plasmin can indeed degrade CMP in an isolated system of enzyme and CMP solution.[18] Lack's investigation of the problem by incubation of cartilage strips with plasmin demonstrated a considerable liberation of CSA as compared to control incubations or those to which antiplasminogen had been added.[8] However, repeated attempts to demonstrate degenerative changes in vivo by multiple injections of plasmin into intact and traumatized animal joints have been singularly unproductive.[11] This paper, however, presents evidence for the presence of an enzyme in normal and osteoarthritic cartilage which may be the agent causing the initial steps in CMP catabolism.

Having demonstrated such an enzyme in both normal and pathological cartilage, the next question which demands a solution is that of the site of action of this enzyme. There are theoretically four potential sites for enzymatic cleavage of this compound whose structure presumably is a linear arrangement of CSA complexes bound to protein.[17] These include 1) proteolysis of

Schubert's protein, 2) depolymerization or further degradation of the polysaccharide, 3) chondrosulfatase activity and 4) cleavage of the protein to polysaccharide bond.

Analysis of osteoarthritic joint fluids reveals fully polymerized CMP staining metachromatically and particulate cartilage containing collagen. Two studies involving an analysis of joint fluids have shown no significant difference in the glucosamine/galactosamine ratio in normal and pathological joint fluids with the exception of one patient in each series who showed an elevated galactosamine. [18,19] Repeated attempts to demonstrate chondrosulfatase activity in cell-free extracts of mammalian connective and parenchymal tissues (costal cartilage, cornea, subcutaneous tissue, liver, kidney and spleen) by in vitro incubation with S^{35} labeled chondroitin sulfate have been singularly unproductive.[20] Studies of the fate of the SO_4 portion subcutaneously injected chondroitin sulfate labeled with S^{35} [20] and 6F S^{35} metabolism utilizing cartilage strip incubation techniques [21] have, however, been suggestive of the presence of such an enzyme. Recent work by Thomas et al, comparing the effects of large doses of vitamin A and of papain has shown a similarity of observable alteration in the cartilage matrix - loss of metachromaticity with an increase of a substance in serum presumed to be chondroitin sulfate because of its turbidimetric characteristics after treatment with hexaminecobaltic chloride and a loss of S^{35} from articular and epiphyseal cartilage.[22] The suggestion was made that vitamin A produced these changes by activation of a proteolytic enzyme in cartilage which had an action similar to papain. The enzyme which has been demonstrated by our work could be responsible for the change produced by large doses of vitamin A. Indeed an intra-

cellular protease with an optimum pH of 3 has been demonstrated in normal chondrocytes which produces changes in the matrix closely resembling those produced by excess vitamin A. (23)

There have been several interesting studies which have investigated the dynamic biochemical changes in osteoarthritic cartilage. Collins and McElligott have shown an increasing avidity for SO_4 in cartilage strips with increasing grade of severity of osteoarthritic changes in the strips and have noted an increase in mucopolysaccharide content of the matrix surrounding all clumps in fibrillated cartilage.(24) McElligott and Potter have reported a 50% greater uptake of S^{35} by papain treated rabbits as compared to controls.(25) These changes, which are interpreted as an attempt at regeneration in injured tissues, are not surprising in view of the demonstration by Crelin and Southwick of mitosis in adult cartilage in response to a pathological stimulus. (26) McElligot and Potter suggested that the overall loss of chondroitin sulfate from the osteoarthritic cartilage is due to the "poor binding power" of the matrix. The enzyme which we have demonstrated in human cartilage may well be responsible for the initial steps in the catabolism of matrix mucopolysaccharide. Although we have not demonstrated a markedly higher activity in osteoarthritic samples as compared to normal controls, the highest activities recorded were in these samples. Fibrillated cartilage showed a much greater activity than either spur or loose body cartilage. The chondrosarcoma was the most active of all samples perhaps reflecting a markedly greater metabolic turnover. We can only infer that this enzyme is responsible for the degenerative changes noted.

Recent work has raised the possibility that this process may be reversible. Chrisman and Southwick in a study of the effects of adjuvants on SO_4 metabolism have noted that incubation of cartilage strips in a medium containing hydluronic acid or chondroitin sulfate increased $S^{35}O_4$ incorporation and total ester sulfate content of the tissue. Incubation with other adjuvants produced a catabolic effect with decrease in total ester sulfate in spite of S^{35} pickup.(27) Recent experiments in this laboratory investigating the effects of salicylate and hydrocortisone on the clearance of $CS^{35}A$ from rabbit knee joints have shown a diminished rate of clearance of S^{35} labeled material from treated as compared to control animals. (28) Perhaps these agents produce a partial reversal or retardation of the degradation of chondromucoprotein by increasing the concentration of reaction products in the joint fluid.

Conclusion

An enzyme has been demonstrated in human cartilage which is capable of degrading chondromucoprotein by splitting off chondroitin sulfate intact. The levels of activity of this enzyme in normal, osteoarthritic, loose body, and chondrosarcomatous cartilage have been investigated. The dynamic biochemical changes in osteoarthritic cartilage are discussed with reference to the possible role of this enzyme in hypertrophic arthritis.

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